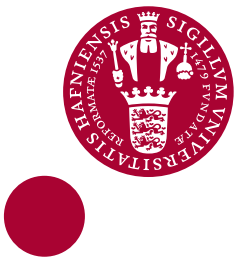

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Uterine expression of smooth muscle alpha- and gamma-actin and smooth muscle myosin in bitches diagnosed with uterine inertia and obstructive dystocia

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ABSTRACT

Primary uterine inertia (PUI) is the most common type of dystocia in dogs. We hypothesized that PUI develops because of lower than normal expression of the basic contractile elements in the uterus, i.e., smooth muscle (SM) α- and γ-actin and SM-myosin, and that the expression of these proteins is influenced by the number of fetuses present *in utero*. Full-thickness inter-placental uterine biopsies were collected during Cesarean sections from dogs with PUI (n = 11), and from bitches with obstructive dystocia (OD) still presenting strong labor contractions (designated as the control group, n = 7). Relative gene expression was determined by semi-quantitative real-time (TaqMan) PCR, and protein localization by immunohistochemistry. Gene expression between PUI and OD bitches, and between PUI bitches carrying small, large, or average number of fetuses according to their breed, were compared. Uterine SM-γ-actin and SM-myosin mRNA levels were significantly higher in PUI than in OD dogs, while SM-α-actin did not differ. PUI bitches carrying large litters had lower uterine SM-γ-actin gene expression than those with small litters (P = 0.008). Immunostaining for SM-actin isoforms and SM-myosin was present in the myometrium, and localization pattern and staining intensity appeared similar in the PUI and OD groups. All proteins stained in blood vessels, and SM-γ-actin was also present in endometrial luminal and glandular epithelium. In conclusion, higher uterine SM-γ-actin and SM-myosin gene expression in PUI bitches, compared with OD dogs, might be an indication of abnormal progression with labor. Whether this is the cause of PUI due to an intrinsic error of the myometrium not becoming committed to labor, or the consequence of inadequate endocrine or mechanical stimuli, is not clear. Litter size was previously shown to be one of the risk factors for the development of uterine inertia in dogs, and our findings suggest possible differing uterine pathophysiology of PUI with respect to litter size.

1. Introduction

Dystocia is a severe problem in the bitch and occurs in 5–16% of all parturitions [1,2]. Predisposed breeds, e.g. French Bulldogs, Boston Terriers, Pugs, Chihuahuas, Bulldogs, Scottish Terriers, Pomeranians, miniature Poodles and Dachshunds have even higher incidence rates [1,3–5]. Maternal causes of dystocia are two to three times more likely than fetal factors, and primary uterine inertia (PUI) accounts for 14–49% of dystocia cases in dogs [3,5,6]. Primary uterine inertia infers an idiopathic, spontaneous inertia, which is defined by the inability of the myometrium to deliver normal sized fetuses by functional contractions through an otherwise normal, unobstructed birth canal [7,8]. However, there are different views regarding subgroups, i.e., total or incomplete PUI, or associated clinical findings [6–15]. To what extent this impedes

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elucidation of the etiology and underlying pathophysiology of PUI remains unclear. There appears to be breed predisposition for PUI in Boxers, Shetland Sheepdogs, Border Collies, Labrador Retrievers and Golden Retrievers [1,3,7,9,16]. Other predisposing factors are older age of the bitch, disproportionally small or large litter size, high body condition score/obesity, and nutritional or hormonal imbalance [7,17,18].

Failure of adequate luteolysis and sustained high maternal peripheral progesterone (P4) concentrations were suspected, but not confirmed in a case study on PUI [19], as all four bitches had P4 concentrations less than 2 ng/mL. Lower plasma prostaglandin F2α metabolite (PGFM) levels and higher P4/PGFM ratio [10], lower total or ionized calcium (iCa) [13,20], higher magnesium [21], lower plasma oxytocin and vasopressin [10,13], and higher serum parathyroid hormone concentrations [20], may contribute to canine uterine inertia, whereas downregulation of uterine oxytocin receptors does not seem to play a role [11]. Genetic predisposition, as well as metabolic defects at the cellular level, were also hypothesized [17], implying direct effects on myometrial smooth muscle cells. Presently, no definite conclusions can be drawn from the few studies investigating the etiology of canine uterine inertia; however, a combination of several endocrine and metabolic alterations seem to be implicated.

Parturition in dogs, similar to other species, is under a complex neuro-hormonal regulation, where oxytocin and PGF2α play essential roles in uterine contractions. The sensitivity of the canine uterus to oxytocin increases during late pregnancy [22], and plasma oxytocin concentrations become elevated and more variable during the expulsive stage of parturition compared to late gestation [23]. Placental PGF2α production results in substantially increased plasma PGFM levels shortly before birth, reaching peak concentrations during stage 2 labor [24,25]. These changes allow for strong, aligned contractions of the myometrium, which is required to guarantee successful expulsion of the fetus(es).

The actomyosin complex has been long known as the functional contractile unit of the uterus [26]. Myosin consists of two pairs of light chains and two heavy chains, with four splice variants of the heavy chain being identified also in the uterus of several species [27,28]. The expression and localization of smooth muscle myosin (SM-myosin) in the canine uterus has not been investigated. Smooth muscle contractions are based on the interaction between actin and myosin, which is regulated by phosphorylation and dephosphorylation of the myosin light chain [29]. Binding of oxytocin or PGF2α to their G-protein coupled receptors on myometrial smooth muscle cells initiates a stimulatory cascade leading to increased free intracellular iCa (from the sarcoplasmic reticulum and from extracellular iCa influx) and the formation of the calcium-calmodulin complex [30–33]. Subsequently, myosin light chain kinase becomes activated by associating with the calcium-calmodulin complex and phosphorylates the regulatory light chain of myosin. This triggers cross-bridge cycling between actin filaments and myosin, i.e., muscle contraction. A decrease in iCa deactivates myosin light chain kinase and allows dephosphorylation of myosin by myosin light chain phosphatase, which causes muscle relaxation [29,33,34]. Smooth muscle cells have four different actin isoforms; it is the amount and ratio of the SM-α-actin and SM-γ-actin isoforms that are reported to depend on the type and physiologic state of the tissue [35–38]. In rats, in contrast to relatively stable myometrial SM-α-actin levels, a progressive rise in SM-γ-actin mRNA and protein expression was noted during pregnancy, with a peak of 31.78-fold increase in mRNA and 16.7-fold increase in protein levels compared to non-pregnant uteri, followed by a slight decrease to 15.21-fold higher than non-pregnant myometrial mRNA levels on the day of labor. This change in SM-γ-actin was due to increased expression in the circular layer of the myometrium [35]. These findings indicate that, in the rat, SM-γ-actin plays an important role during pregnancy as well as in preparation of the myometrium for contractions during labor, which may also be true for other species, such as the dog. While uterine expression and localization of SM-γ-actin in bitches has not yet been described, SM-α-actin immunoreactivity was found, not only in the myometrium and vascular smooth muscle cells of non-pregnant dogs [39], but also in uterine stromal cells during the pre-implantation period [40]. Furthermore, SM-α-actin was strongly induced in maternal decidual cells later in gestation [40], supporting its role in canine pregnancy.

To date, the contractile apparatus of the canine uterus has not been characterized so as to fully understand the etiology of decreased myometrial contractility in PUI. The goal of our study was to investigate the localization and expression of SM-α- and SM-γ-actin and SM-myosin in the uterus of parturient bitches diagnosed with PUI by history, clinical signs, imaging and tocodynamometry [11,13,14,41], and to compare them with bitches without myometrial dysfunction during whelping. We hypothesized that uterine tissue expression of SM-α- and SM-γ-actin and SM-myosin may be decreased and/or the localization pattern of these proteins altered in dogs with PUI, compared to bitches showing normal labor contractions.

2. Material and methods

2.1. Animals and collection of uterine biopsy samples

Bitches presented with dystocia, and diagnosed with PUI or obstruction treated with an emergency Cesarean section (CS), were included in this study, provided they did not receive any ecbolic (oxytocin, calcium) or tocolytic medication before sampling. All CS were carried out only if medically indicated. A detailed general and reproductive history was taken for all dogs, general clinical and obstetrical examinations were carried out, body weight and body condition score (BCS) were recorded, and further diagnostics (e.g. bloodwork, diagnostic imaging) were performed as deemed necessary.

The criteria used for categorizing bitches into the PUI or obstructive dystocia (OD) groups were as described in our previous work [41]. Although bitches showing adequate labor contractions and giving birth to one or more puppies have been previously classified into PUI (partial or incomplete [6,8,13]), we only grouped bitches into PUI if they had not given birth to any puppies. To be assigned to the PUI group, they must have been in first stage labor for $\geq$ 20 h, or shown passage of fetal fluids or green vaginal discharge $\geq$ 2–3 h previously without any visible abdominal contractions and progress into adequate second stage labor, or had only weak, infrequent abdominal contractions for >4 h. Obstetrical evaluation was performed at presentation during dystocia, and this included palpation of the vagina and imaging in all cases. Additionally, tocodynamometry was used for 20 min if the dam's clinical condition was stable, and if fetal heart rates were not reduced. PUI was diagnosed if there was no, or very weak abdominal straining in response to digital vaginal stimulation to induce uterine contractions, and/or weak, infrequent, or no uterine contractions measured with external tocodynamometry. Obstruction of the birth canal was excluded by digital vaginal palpation and/or abdominal radiographs. The control group consisted of bitches presented with obstruction (OD group), which was diagnosed based on radiographs and/or by vaginal palpation. To achieve the goal of our study, i.e., to compare representative uterine tissues from bitches with good, physiologic uterine contractility to those with PUI, only dogs displaying strong spontaneous abdominal straining, as well as strong abdominal and uterine contractions in response to digital vaginal stimulation, were included in the OD group. Bitches with signs of systemic illness (e.g.

sepsis, metritis) or other causes that could contribute to uterine inertia development (e.g. uterine torsion, rupture) were not included in the study.

The PUI group (n = 11) consisted of two Maremma Sheepdogs, two Maltese, and one each of German Shepherd, Broholmer, Labrador Retriever, Beagle, French Bulldog, Dachshund and Boxer. The OD group (n = 7) was represented by two Chihuahuas and one each of Cairn Terrier, Yorkshire Terrier, West Highland White Terrier, Staffordshire Bullterrier and one medium size mixed breed dog.

Uterine tissue biopsies were collected during CS after all puppies had been delivered. At the site of the hysterotomy incision, one full thickness uterine tissue sample was excised from the interplacental (IPL) uterus (between two placentation sites). In bitches undergoing concurrent ovariohysterectomy at the time of CS, samples were taken after the uterus was removed.

For immunohistochemistry (IHC), IPL tissues were fixed for 24 h at 4 °C in 10% neutral phosphate-buffered formalin, washed in frequently changed phosphate-buffered saline, dehydrated in a graded ethanol series and xylol, and embedded in paraffin. For preservation of RNA, tissues were incubated for 24 h in RNAlater® (Thermo Fisher Scientific, Waltham, MA, USA) at 4 °C, and later stored at −80 °C until use.

Owners signed an informed consent for uterine biopsy sample collection. The study was approved by the respective authorities (Cantonal Veterinary Office Zurich, permit no. ZH086/15; Dyreforsøgstilsynet Fødevarestyrelsen, Denmark, permit no. 2015-15-0201-00513).

2.2. Total RNA extraction and reverse transcription

TRIzol® reagent (Invitrogen, Carlsbad, CA, USA) was used to isolate total RNA from IPL uterine tissue samples, according to a previously described protocol [42]. The extracted total RNA concentration was measured on a NanoDrop 2000C® spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). To eliminate genomic DNA contamination, 200 ng total RNA per sample and target gene was treated with RQ1 RNase-free DNase (Promega, Dübendorf, Switzerland), according to the manufacturer's protocol. Reverse transcription was carried out using random hexamers and reagents from Applied Biosystems (Foster City, CA, USA), according to our protocol [42].

2.3. Molecular cloning of canine SM-α-actin, SM-γ-actin, and SM-myosin heavy chain 11

To date, only predicted sequences are available for canine-specific SM-α-actin (ACTA2, GenBank accession no. XM_534781.6), SM-γ-actin (ACTG2, GenBank accession no. XM_533002.5) and SM-myosin (SM-myosin heavy chain 11, MYH11, GenBank accession no. XM_005621514.2). Therefore, we performed molecular cloning and sequencing of the respective genes to confirm their identity using canine-specific primers (Microsynth AG, Balgach, Switzerland), which are shown in Table 1. The cloned partial sequence of SM-myosin is common in all four predicted canine sequence variants. Briefly, GeneAmp Gold RNA PCR Kit (Applied Biosystems) was used in a hot-start PCR, according to our protocol [42,43]. The annealing temperature was 58 °C for all three genes. Amplification of cDNA, corresponding to 200 ng DNase-treated total RNA from two uterine samples, yielded PCR products of 676bp, 669bp and 598bp, corresponding to partial canine sequences of SM-α-actin, SM-γ-actin and SM-myosin, respectively (Table 1). Autoclaved water, instead of cDNA, was used as a negative control as well as an RT-minus control, in which no RT reaction was carried out. PCR products were separated on a 2% ethidium bromide-stained agarose gel, and were extracted using a Qiaex II gel extraction system (Qiagen GmbH). Subsequently, they were cloned into pGEM-T vector (Promega) and transformed and amplified in XL1 Blue competent cells (Stratagene, La Jolla, CA, USA). Plasmid DNA was purified with Pure Yield Plasmid MidiPrep System (Promega) and sequenced on both strands with T7 and Sp6 primers (Microsynth). Sequence analysis showed 99% identity of the cloned sequence to the predicted canine sequence of SM-α-actin, and 100% homology between the cloned and predicted canine sequences of SM-γ-actin and SM-myosin heavy chain 11. Partial CDs have been submitted to GenBank with accession number MN968927 for SM-α-actin (ACTA2), MN968928 for SM-γ-actin (ACTG2), and MN968929 for SM-myosin (MYH11).

2.4. Semi-quantitative real-time (TaqMan) PCR

Semi-quantitative real-time (TaqMan) PCR was performed using an automated fluorometer from Applied Biosystems (ABI PRISM$_{TM}$ 7500 Sequence Detections System, Foster City, CA, USA), according to our previously described protocol [44]. Fast Start Universal Probe Master (ROX®) (Roche Diagnostics AG, Schweiz) was used. All samples were run in duplicates. Autoclaved water and the RT-minus control (without RT reaction) served as negative controls. Canine-specific primers and hydrolysis (TaqMan) probes are listed in Table 1. Hydrolysis (TaqMan) probes were labeled at the 5′-ends with the reporter dye, 6-caroxyfluorescein (FAM), and at the 3′-ends with the quencher, 6-carboxytetramethyl-rhodamine (TAMRA). Canine glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*, GenBank: AB028142) [42]; Microsynth, Balgach, Switzerland) and *cyclophyllin A* (Prod. No. Cf03986523-gH, Applied Biosystems, Foster City, CA, USA) were used as reference genes. Efficiency of the PCR reactions was ~100%, which was calculated with the CT slope method according to the manufacturer's instructions of the ABI PRISM$_{TM}$ 7500 Sequence Detection System, and as previously described [45]. Selected PCR products were sent for sequencing (Microsynth).

Relative gene expression (RGE) was calculated based on the comparative CT method (ΔΔCT method), according to the manufacturer's protocol and our previous reports [42,46]. The sample with the lowest detectable amount of transcript was used as the calibrator.

2.5. Immunohistochemistry

We used a previously described indirect immunoperoxidase method for protein detection and localization in uterine tissues [40,42]. Formalin-fixed IPL uterine samples were embedded in paraffin, cut into 2–3 μm slices using a microtome, and mounted on microscope slides (SuperFrost, Menzel-Glaeser, Braunschweig, Germany) before being deparaffinized in xylol and rehydrated in a graded ethanol series. Antigen retrieval was performed in 10 mM citrate buffer (pH 6.0) at 100 °C for 15 min. Tissue samples were allowed to cool, and then they were put into 0.3% hydrogen peroxide in methanol for 30 min to stop endogenous peroxidase activity. After being washed in IHC buffer solution (0.3% Triton X, pH 7.2–7.4; 0.8 mM Na_2HPO_4, 1.47 mM KH_2PO_4, 2.68 mM KCl, 137 mM NaCl), nonspecific binding sites were blocked with 1.5% bovine serum albumin and 10% goat serum (for SM-myosin and SM-γ-actin) or 10% horse serum (for SM-α-actin). Incubation with the respective antibodies was carried out overnight at 4 °C. The following antibodies and dilutions were used: Anti-smooth muscle myosin heavy chain 11 (Abcam, Cambridge, UK; polyclonal rabbit IgG; 1:500), anti-human smooth muscle α-actin (DAKO, Denmark A/S; monoclonal mouse IgG2a; 1:800), and anti-smooth muscle γ-actin (AVIVA Systems Biology, San Diego, CA, USA; polyclonal rabbit IgG; 1:1500). The antibody used for SM-myosin heavy chain 11 detects all heavy chain isoforms. After washing the slides with IHC

Table 1
List of canine-specific primers used for molecular cloning, and canine-specific primers and TaqMan probes used for semi-quantitative real-time PCR.

Gene	Application	Primer and TaqMan probe sequences	Amplicon length	GenBank Accession no.
SM-α-actin (*ACTA2*)	Cloning	Forward primer: 5′- TAG AAC ACG GCA TCA TCA CC – 3′ Reverse primer: 5′- TTG GCG TAC AGG TCT TTC CT – 3′	676 bp	XM_534781.6
	qPCR	Forward primer: 5′- ACA CGG CAT CAT CAC CAA CTG – 3′ Reverse primer: 5′- CAG GGT GGG ATG CTC TTC GG – 3′ TaqMan probe: 5′- TTT CTA CAA CGA GCT CCG TGT CGC CC – 3′	97 bp	
SM-γ-actin (*ACTG2*)	Cloning	Forward primer: 5′- ATT CAA GCC GTG CTG TCT CT – 3′ Reverse primer: 5′- GCT GAT CCA CAT CTG CTG AA – 3′	669 bp	XM_533002.5
	qPCR	Forward primer: 5′- GCC GTG ACC TCA CTG ACT ACC – 3′ Reverse primer: 5′- CCA GGG CCA CAT AGC ATA GCT T – 3′ TaqMan probe: 5′- TCG CAC AAT TTC TCG CTC AGC TGT GGT – 3′	120 bp	
SM-myosin (heavy chain 11, *MYH11*)	Cloning	Forward primer: 5′- CGC CAC ATC TCA ACT CTG AA – 3′ Reverse primer: 5′- GCT TGG CAT CTT CTG TAG CC – 3′	598 bp	XM_005621514.2
	qPCR	Forward primer: 5′- GGC TTC AGC AGG AGC TGG AC – 3′ Reverse primer: 5′- TCT CCT CAG CCA ACA ACT GAT CG – 3′ TaqMan probe: 5′- ACC AGC GGC AAC TGG TGT CCA ACC – 3′	111 bp	
GAPDH	qPCR	Forward primer: 5′-GCT GCC AAA TAT GAC GAC ATC A-3′ Reverse primer: 5′-GTA GCC CAG GAT GCC TTT GAG-3′ TaqMan probe: 5′-TCC CTC CGA TGC CTG CTT CAC TAC CTT-3′	75 bp	AB028142

buffer solution to remove unbound primary antibody, a biotinylated secondary goat anti-rabbit IgG antibody (Vector Laboratories, Burlingame, CA, USA) for SM-myosin and SM-γ-actin, and a horse anti-mouse IgG (Vector Laboratories, Burlingame, CA, USA) for SM-α-actin, were applied for 30 min at a dilution of 1:100. In the next step, an avidin/biotinylated peroxidase complex (Vectastain ABC Kit, Vector Laboratories, Burlingame, CA, USA) was used for 30 min to enhance signals. Color reaction was achieved by applying 3,3′-diaminbenzidine (Liquid DAB + substrate Kit, Dako Schweiz AG, Baar, Switzerland). Finally, slides were counterstained with Mayer's hematoxylin and dehydrated in a graded ethanol series. The following isotype controls were used for each antibody as negative controls: For SM-α-actin, the primary antibody was replaced with pre-immune mouse IgG2a (EXBIO Praha a.s., Vestec, CZ), and for SM-γ-actin and SM-myosin heavy chain 11, pre-immune rabbit IgG was used (Vector Laboratories, Burlingame, CA, USA). IHC slides were assessed qualitatively.

2.6. Statistical analysis

Data were evaluated using IBM SPSS® Statistics for Windows, Version 24.0 (Armonk, NY, USA) program package. Body weight (BW), body condition score, age, current pregnancy number, and litter size were compared between dystocia groups (PUI and OD) using a *t*-test or a Mann-Whitney *U* test. Univariate ANOVA was used to compare relative gene expression (RGE) of SM-α-actin, SM-γ-actin, and SM-myosin in the IPL uterine tissues between the PUI and OD groups. Group was used as the fixed factor, and because BW of the bitches in the two dystocia groups was significantly different, BW was added as covariate in the analysis to account for that difference.

Afterwards, the PUI group was divided into three subgroups based on their litter size relative to the breed average litter size [47]. Normal litter size (PUI–N) was defined, when the number of puppies born was within ±1 standard deviation (SD) of the breed average. Small litter size (PUI–S) was defined as below -1SD of the breed average, and large litter size (PUI-L) was defined as above +1SD of the breed average. Gene expression within these PUI subgroups was analyzed with univariate ANOVA using subgroup as the fixed factor followed by Tukey Honestly Significant Difference. BW was similar among the PUI subgroups ($P = 0.639$, univariate ANOVA) and therefore not included in this analysis.

Lastly, taking all participating bitches into account (PUI and OD together), Pearson correlation was used to determine the associations among the expression of the respective genes. Level of significance for all analyses was set at $P < 0.05$.

3. Results

3.1. Animals and groups

Mean body weight of bitches in the PUI group was higher than that in the OD group ($P = 0.017$; Table 2). No difference in body condition score, age, current pregnancy number or litter size was found between the two dystocia groups ($P \geq 0.220$; Table 2). Four dogs in the PUI group had smaller than breed average litter size (Dachshund, French Bulldog, Boxer, Maremma Sheepdog), four had average litter size (Maltese, German Shepherd, Labrador Retriever, Broholmer), and three had more than the breed average number of puppies (Maltese, Beagle, Maremma Sheepdog). All dogs in the OD group had litter size according to breed average, except one Chihuahua bitch with only two puppies.

3.2. Gene expression of SM-α-actin, SM-γ-actin and SM-myosin in interplacental uterine tissues

Gene expression of SM-γ-actin and SM-myosin in the uterus was higher in the PUI group than in the OD group ($P = 0.006$ and $P = 0.018$, respectively), while there was no difference in SM-α-actin mRNA levels (Fig. 1).

Comparing PUI subgroups, which were created according to litter size, a significant difference was found in the relative gene expression of SM-γ-actin, but not in SM-α-actin and SM-myosin ($P = 0.269$ and $P = 0.385$, respectively; Fig. 2). Bitches with PUI carrying more puppies than the breed average had lower uterine SM-γ-actin mRNA expression than dogs with a smaller than average litter size ($P = 0.008$).

Analyzing all bitches together, moderate to strong positive correlations (Fig. 3) were detected between uterine mRNA concentrations of SM-α-actin and SM-γ-actin ($r = 0.674$, $P = 0.001$), and between SM-myosin and both actin isoforms (SM-γ-actin, $r = 0.747$, $P < 0.0001$; SM-α-actin, $r = 0.708$, $P = 0.001$).

3.3. Immunohistochemical detection of SM-α-actin, SM-γ-actin and SM-myosin in interplacental uterine tissues

Immunostaining for both SM-actin isoforms (Figs. 4 and 5) and for SM-myosin (Fig. 6) was detected in the circular and longitudinal

Table 2
General and reproductive characteristics of bitches in the study.

Dystocia groups	N	BW (kg)	BCS	Age (years)	Current pregnancy number	Litter size
PUI	11	28.26 ± 20.14[a] (5.6–70.1)	4.43 ± 0.98[a] (3–6)[#]	4.45 ± 1.94[a] (1.9–7.1)	2.1 ± 0.99[a] (1–4)[§]	5.64 ± 3.07[a] (2–9)
OD	7	9.26 ± 6.67[b] (2.9–21.2)	4.33 ± 0.82[a] (3–5)[§]	3.13 ± 2.43[a] (0.9–8.1)	1.57 ± 0.79[a] (1–3)	4.29 ± 2.06[a] (2–8)

[a,b] different superscripts in a column denote significant differences between dystocia groups; [#]data not available for three animals; [§]data not available for one animal; PUI: primary uterine inertia; OD: obstructive dystocia; BW: body weight; BCS: body condition score on a 9-point scale [48]. Mean ± standard deviation and minimum-maximum in parentheses are shown.

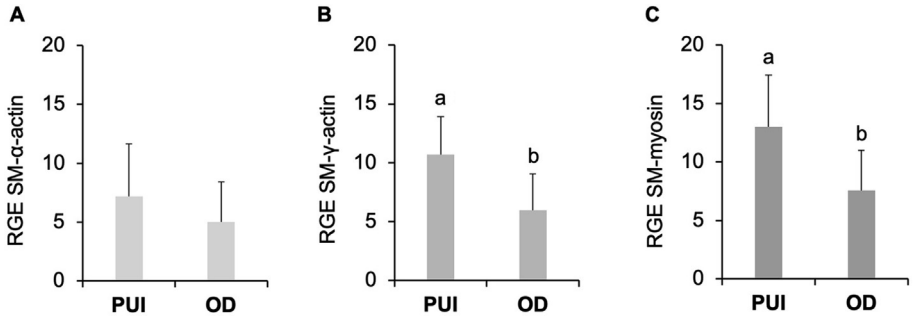

Fig. 1. Relative gene expression (RGE) of A) smooth muscle α-actin (SM-α-actin), B) smooth muscle γ-actin (SM-γ-actin), and C) smooth muscle myosin heavy chain 11 (SM-myosin) as determined by semi-quantitative real-time (TaqMan) PCR in interplacental uterine tissue homogenates of bitches in the primary uterine inertia (PUI) and obstructive dystocia (OD) groups. Bars denote the mean and whiskers denote the standard deviation. Different letters (a,b) denote significant ($P < 0.05$) differences between the groups as evaluated by ANOVA with body weight included as covariate.

layers of the myometrium in both the PUI and OD groups. Beside diffuse cytoplasmic signals, a strong, round staining pattern was visible under the plasma membrane of some of the myometrial smooth muscle cells for all proteins (Figs. 4C, 5C and 6C). We did not observe a difference in localization pattern or staining intensity between the PUI and OD groups, or between PUI subgroups, in regard to SM-α- and -γ-actin and SM-myosin. However, there were individual differences in staining intensity. Positive immunoreactivity was found for all proteins in the tunica media of blood vessels (Figs. 4–6), and in the basement membrane of blood vessels for SM-α-actin and SM-γ-actin (Figs. 4 and 5). SM-γ-actin protein was also expressed in luminal and glandular epithelial cells of the endometrium (Fig. 5D).

4. Discussion

All contractile proteins in our study, i.e., SM-α-actin, SM-γ-actin, and SM-myosin, were predominantly localized in the myometrium, confirming their role in uterine smooth muscle contractions in the dog. Not surprisingly, given that SM-actin and SM-myosin interactions are ultimately responsible for muscle contractions [29,33,34], their mRNA concentrations showed strong correlations. Furthermore, the presence of distinct immunoreactive areas, which we observed for all proteins close to the plasma membrane in some myometrial smooth muscle cells, might be a common feature of the parturient canine uterus, similarly to what had been described in late pregnant rats [35].

Differences in gene expression of SM-γ-actin and SM-myosin between PUI and control OD bitches support our hypothesis of an altered uterine environment, which may be responsible for inadequate uterine contractions in PUI cases. Finding higher mRNA concentrations of these contractile proteins in dogs with uterine inertia seems at first conflicting, assuming that a more abundant contractile machinery would be capable of stronger contractions. The myometrium undergoes substantial morphologic and biochemical changes during pregnancy, by the end of which it acquires the laboring phenotype, enabling the dam to undergo parturition [49]. Accordingly, the abundance of the actomyosin complex, the primary contractile element in the uterus, increases during pregnancy, reaching maximum levels at the time of labor,

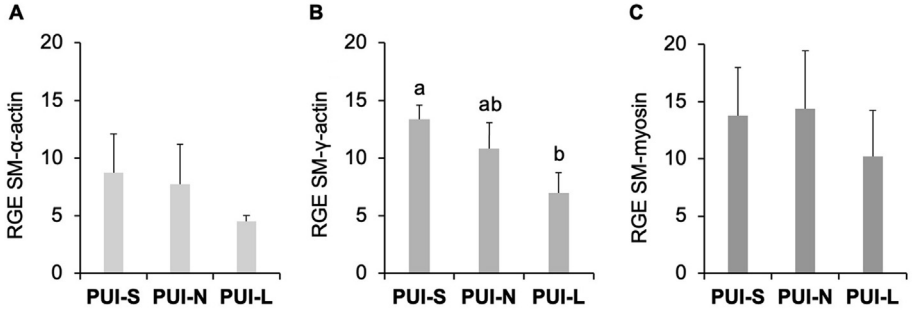

Fig. 2. Relative gene expression (RGE) of A) smooth muscle α-actin (SM-α-actin), B) smooth muscle γ-actin (SM-γ-actin), and C) smooth muscle myosin heavy chain 11 (SM-myosin) as determined by semi-quantitative real-time (TaqMan) PCR in interplacental uterine tissue homogenates of bitches in the primary uterine inertia (PUI) group with different litter sizes. PUI-S stands for small, PUI-N for normal (average) and PUI-L for large litter size. Litter size classification was done according to the average of the breed [47]. Bars denotes the mean and whiskers the standard deviation. Different letters (a, b) denote significant ($P < 0.05$) differences between the groups as evaluated by ANOVA.

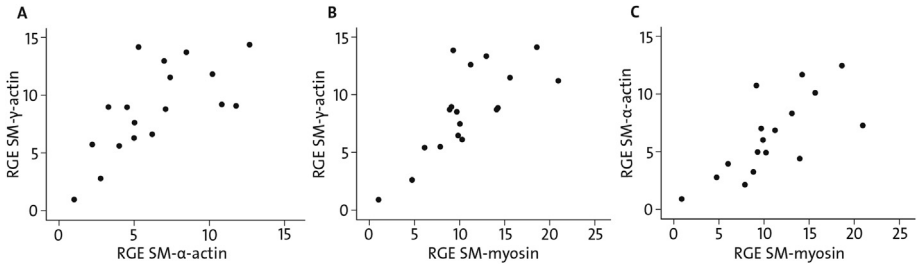

Fig. 3. Correlation of relative gene expression (RGE) of A) smooth muscle α-actin (SM-α-actin) and smooth muscle γ-actin (SM-γ-actin) ($r = 0.674$, $P = 0.001$), B) smooth muscle myosin heavy chain 11 (SM-myosin) and SM-γ-actin ($r = 0.747$, $P < 0.0001$), and C) SM-myosin and SM-α-actin ($r = 0.708$, $P = 0.001$) in interplacental uterine tissue samples of all bitches (primary uterine inertia and obstructive dystocia groups together).

and decreases quickly after parturition [26]. In rats, myometrial SM-γ-actin mRNA expression slightly decreased from peak levels on day 19 of pregnancy to the day of labor, and then dropped significantly by day 1 postpartum [35], which is likely a physiologic process. If we assume that SM-γ-actin expression also decreases from the end of pregnancy through successful labor in the uterus of bitches, similar to rats, then the myometrium of dogs belonging to the PUI group did not progress to an adequate laboring phenotype. In contrast, dogs in the OD group, which presented with strong uterine contractions consistent with normal second stage labor, had significantly lower SM-γ-actin and SM-myosin gene expression levels. Whether this is the reason or the consequence of abnormal labor in PUI bitches is unclear. Quantification of protein expression (by Western blotting) might provide additional insights into the role of the respective proteins. Similar to our findings, the cause and effect dilemma was also noted in a recent study, which investigated uterine oxytocin receptor mRNA expression in the etiology of canine dystocia. After a physiologic upregulation near term [11,22,50], down-regulation of uterine oxytocin receptors was shown to occur with prolonged labor [11]. However, in bitches with

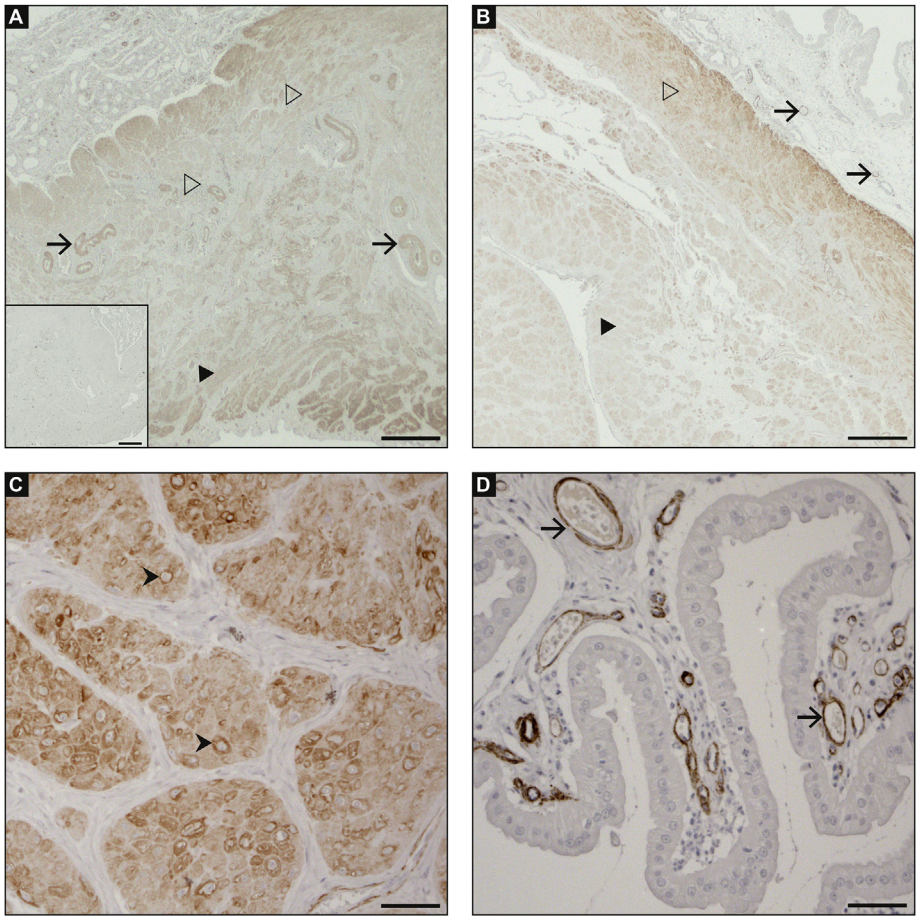

Fig. 4. Immunohistochemical localization of smooth muscle α-actin in a representative interplacental uterine tissue sample from A) the primary uterine inertia group and B) the obstructive dystocia group. In both groups, positive immunoreactivity for smooth muscle α-actin is visible in the longitudinal (▶) and circular (▷) layer of the myometrium. Signals were also detected in blood vessels (→) in the tunica media and basement membrane. Inset shows the isotype control. C) Smooth muscle cells in the myometrium show diffuse immunostaining in the cytoplasm, while in some cells, strong immunoreactivity appearing as a ring in the area under the plasma membrane, is visible (➤). D) In the endometrium, positive smooth muscle α-actin immunoreactivity was detected in blood vessels (→). A,B: Scale bar = 400 μm. C,D: Scale bar = 50 μm.

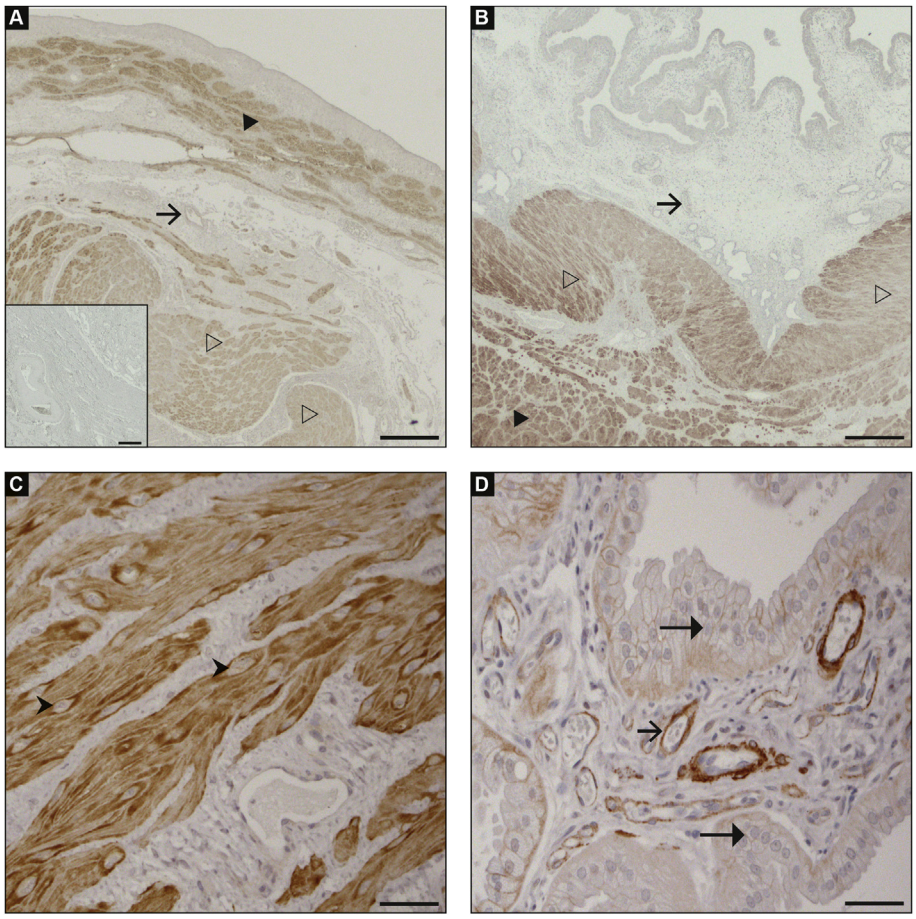

Fig. 5. Immunohistochemical localization of smooth muscle γ-actin in a representative interplacental uterine tissue sample from A) the primary uterine inertia group and B) the obstructive dystocia group. Immunoreactivity for smooth muscle γ-actin is visible in the longitudinal (▶) and circular (▷) layer of the myometrium in both groups. Positive staining is also detectable in the tunica media and basement membrane of blood vessels (→). Inset shows the isotype control. C) Smooth muscle cells in the myometrium show diffuse immunostaining in the cytoplasm, while in some cells, strong immunoreactivity, appearing as a ring in the area under the plasma membrane, is visible (➤). D) In the endometrium, smooth muscle γ-actin immunoreactivity was noted in luminal (➡) and glandular epithelial cells at the plasma and basement membrane, and in blood vessels (→). A,B: Scale bar = 400 μm. C,D: Scale bar = 50 μm.

complete PUI, uterine oxytocin receptor gene expression showed a large variation and did not differ from the other groups, i.e., partial PUI, or bitches undergoing elective CS showing upregulation, or those with obstructive dystocia showing downregulation [11]. Overall, at this point, it appears plausible that uterine inertia either develops because of inadequate progression of uterine responses to normal or abnormal parturition signals, or because of *de novo* abnormal expression of contractile and contractility-associated proteins in the uterus, or as a result of both.

Next, we wanted to prove that litter size accounts for different pathogenesis of PUI, e.g. possible suboptimal stimulation in the case of a small litter vs. overstretching of the myometrium in the case of a large litter. Therefore, we compared uterine expression of the smooth muscle actin isoforms and SM-myosin across the three PUI groups. Although one might expect higher expression of each contractile element in dogs with large litter size as their uteri have to be prepared for a longer, and perhaps more exhausting labor period, an inverse relationship between SM-γ-actin and litter size was found at the mRNA level. This finding may indicate a more advanced timeline of labor events in PUI-L bitches. Nevertheless, a larger number of fetuses and a more distended uterus could have influenced other endocrine, biochemical, or mechanical pathways not investigated here, which decrease or inhibit uterine contractions. In contrast to PUI-L dogs, higher uterine SM-γ-actin gene expression in PUI-S bitches could be the sign of insufficient progress with normal second stage labor.

Our study was performed in a real clinical setting. However, including dogs of various breeds and sizes presented for dystocia in emergency might hinder the meaningfulness of results. To reduce this limitation of our study design, we applied strict criteria not only to patient selection, i.e., exclusion of bitches that received medications, but also to inclusion in the PUI or OD groups, although this made finding enough affected individuals time-consuming. Furthermore, to account for at least some aspects of this diversity, we included body weight in the statistical evaluation.

Our PUI diagnosis was made in a similar fashion to previous reports in clinical settings [10,11,13], although ideally, a combination of history, clinical findings and specific measurements performed in defined time windows, e.g. Doppler sonography for uterine perfusion [51,52], degree of cervical dilation, and/or tocodynamometry from before stage 1 labor [19] should be used to classify cases into PUI or PUI subgroups (i.e., total or incomplete). Serial tocodynamometry recordings starting already one week before the expected day of whelping in bitches entering breeding facilities [19] might be a superior method, but it is not available in all countries. At home tocodynamometry monitoring may be beneficial for individual breeders, if performed under close veterinary supervision to facilitate dystocia recognition and admittance

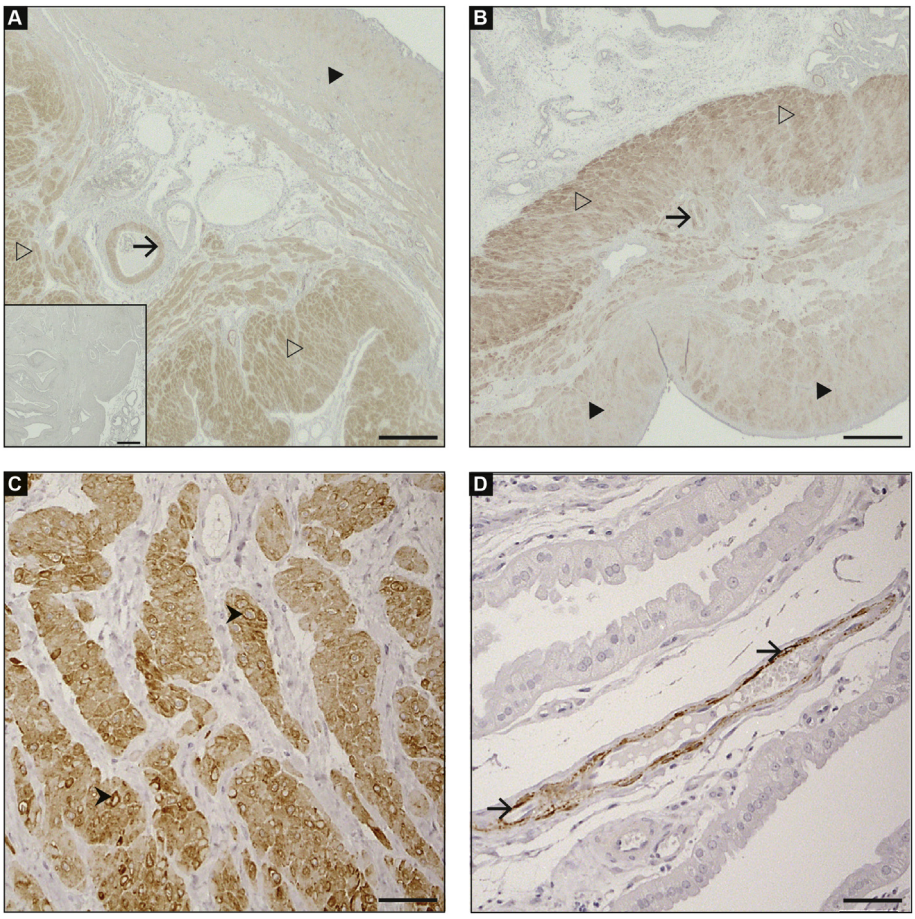

Fig. 6. Immunohistochemical localization of smooth muscle myosin (heavy chain 11) in a representative interplacental uterine tissue sample from A) the primary uterine inertia group and B) the obstructive dystocia group. Staining for smooth muscle myosin heavy chain 11 is detectable in the longitudinal (▶) and circular (▷) layer of the myometrium, and in the tunica media of blood vessels (→) in both groups. Inset shows the isotype control. C) Smooth muscle cells in the myometrium show diffuse immunostaining in the cytoplasm, while in some cells, strong immunoreactivity appearing as a ring in the area under the plasma membrane, is visible (➢). D) In the endometrium, only blood vessels had positive signals. A,B: Scale bar = 400 μm. C,D: Scale bar = 50 μm.

of patients to veterinary hospitals if treatment becomes necessary. In women, electrohysterography or intrauterine pressure recordings more reliably reflect uterine activity [53–55]; however, they are not yet established in the dog. As most bitches with PUI (total or incomplete) will undergo CS, with or without prior medical treatment attempts [6,13,56], it has long been desired to seek the cause of, as well as potential new therapies for, canine uterine inertia [9]. Our goal to advance canine obstetrics is similar to that of human medicine, i.e., "Future research to enhance our understanding of optimal contractile activity and the causes of ineffectual contractions is necessary as we seek to lower rates of cesarean deliveries" [53].

5. Conclusion

In conclusion, we found that the myometrium of parturient bitches expresses both SM-actin isoforms (α and γ), as well as SM-myosin (heavy chain 11). Uterine gene expression of these major contractile elements showed strong correlations, pinpointing their concerted function in contractility. We could not confirm our hypothesis that inadequate uterine contractions in PUI dogs are a consequence of decreased uterine expression of these contractile proteins. Instead, we found that PUI bitches had significantly higher inter-placental uterine SM-γ-actin and SM-myosin gene expression compared to OD bitches, which is likely an indication of their abnormal progression with labor. Whether this is the cause of uterine inertia due to an intrinsic error of the myometrium not becoming fully committed to labor, or the consequence of inadequate or a lack of endocrine or mechanical stimuli, is not yet clear, and needs further investigation. Litter size was previously shown to be one of the risk factors for the development of uterine inertia in dogs [6,7], and we hypothesized that the expression of the contractile elements in the uterus varies with litter size. Our data supports this assumption by showing that bitches carrying smaller or larger than breed average number of fetuses differ in their uterine gene expression of SM-γ-actin, which underscores the possible difference in uterine pathophysiology of PUI with respect to litter size.

CRediT authorship contribution statement

S. Egloff: Investigation, Data curation, Formal analysis, Writing - original draft. **I.M. Reichler:** Conceptualization, Funding acquisition, Investigation, Writing - review & editing. **M.P. Kowalewski:** Investigation, Methodology, Writing - review & editing. **S. Keller:** Investigation, Writing - review & editing. **S. Goericke-Pesch:** Conceptualization, Funding acquisition, Data curation, Investigation, Writing - review & editing. **O. Balogh:** Conceptualization, Funding acquisition, Investigation, Data curation, Formal analysis, Writing - original draft.

Declaration of competing interest

None.

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